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Divergent Regulation of GAP-43 Expression and CNS Neurite Outgrowth by Cyclic AMP

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Robust process outgrowth and high expression of the growth-associated protein GAP-43 seem to be intrinsic features of neurons, but both are down-regulated after axonal contact of target cells. We report that chronic exposure of the serotonergic CNS cell line RN46A to cyclic AMP analogs, forskolin, or cholera toxin represses GAP-43 expression in a dose dependent manner. Thus, cAMP could mediate a GAP-43 repressive signal that is initiated extracellularly. Activation of the cyclic AMP pathway by these same reagents, however, enhances the rate that RN46A cells extend neurites. This stimulation of neurite growth can occur during inhibition of new transcription, and in the absence of high levels of GAP-43. These findings demonstrate that a GAP-43-repressing intracellular signaling pathway exists, that repression of GAP-43 expression by cAMP is not directly coupled to inhibition of neurite growth, and that acceleration of growth cone advancement by cAMP is not dependent on the presence of GAP-43. J. Neurosci. Res. 61:626–635, 2000.

Key words: neuron; axon growth; gene regulation, RN46A cells

Process outgrowth is one of the earliest features of newly differentiated neurons, and is accompanied by high expression of GAP-43. Robust process outgrowth is also exhibited by neurons in culture, where extrinsic influences can be minimized, suggesting that process outgrowth is a default phenotype of neurons. When growing axons reach their targets in vivo, growth cone morphology and function are lost, synaptic structures are created, and the parent neurons alter their pattern of protein synthesis, including a reduction in synthesis of GAP-43.

Target derived signals are thought to play a role in retrogradely regulating neuronal phenotype as neurons establish contact with their targets (Campbell and Frost, 1987; Schotzinger and Landis, 1988; Catsicas et al., 1991; Erzurumlu et al., 1993). Several aspects of the mature phenotype are lost after axon transection in the adult (Goldstein et al., 1988; Oblinger and Lasek, 1988; Bisby and Tetzlaff, 1992; Wu et al., 1993), a procedure that would eliminate any retrograde, target-derived signals. Although little is known concerning the nature of target-derived maturation signals, the neurotrophins NGF and NT3 can reverse some of the effects of axotomy in sensory neurons (Fitzgerald et al., 1985; Verge et al., 1990, 1995, 1996), suggesting that these molecules may be target derived factors involved in the generation of a mature neuronal phenotype.

The growth-associated protein GAP-43 is an axonally transported protein that is expressed at high levels during developmental axon growth or after adult injury in association with regenerative growth. It is localized predominately at the distal axon, and is thought to play a role in signal transduction at the growth cone (Skene, 1989; Strittmatter et al., 1992; Benowitz and Routtenberg, 1997). In studies of adult dorsal root ganglion (DRG) neurons it has been postulated that the expression of GAP-43 is under the control of a peripheral target derived repressing influence because GAP-43 expression increases only after peripheral, and not central axotomy (Schreyer and Skene, 1993). Further support for this concept comes from observations that the magnitude of axotomy-induced GAP-43 up-regulation is independent of lesion distance from the cell soma (Liabotis and Schreyer, 1995), that elevation of GAP-43 is prolonged if peripheral reinnervation is prevented (Bisby, 1988; Schreyer and Skene, 1991), and that disruption of axonal transport mimics injury in inducing up-regulation of GAP-43 (Woolf et al., 1990). No target derived repressors of GAP-43 expression have yet been identified.

Studies of DRG neurons indicate that activation of cyclic adenosine monophosphate (cAMP) signaling can block an increase in GAP-43 expression that occurs when adult neurons are explanted to tissue culture (Schreyer et al., 1997; Andersen et al., 2000), suggesting that this second messenger may be involved in mediating some extracellular GAP-43 repressing signal. In the present study we examine both GAP-43 expression and neurite outgrowth using a cell line derived from embryonic CNS

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neurons. This model allows us to broaden our analysis from the PNS to the CNS, and to eliminate confounding factors associated with the presence of multiple cell types in culture.

RN46A cells are a conditionally immortalized cell line derived from embryonic rat raphe nucleus neuron precursors (White et al., 1994). A temperature-sensitive mutant of the SV40 large T-antigen drives proliferation of these cells at permissive temperature (33°C). At non-permissive temperature (39°C) these cells cease proliferating and adopt a neuronal phenotype that includes extension of long processes. Activation of cAMP signaling in RN46A cells has previously been demonstrated to increase synthesis of neurofilament proteins, tryptophan hydroxylase, and serotonin reuptake transporters (White et al., 1994, 1997; Eaton and Whittemore, 1995) suggesting the hypothesis that cAMP causes these neurons to switch to a more mature phenotype. We therefore sought to determine if cAMP would also act to repress two features of the immature neuronal phenotype, high GAP-43 expression and the propensity to extend long neurites.

MATERIALS AND METHODS

Reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Procedures were carried out at room temperature, unless otherwise noted.

Cell Culture

RN46A cells (White et al., 1994) were cultured on Nunc or Falcon tissue culture plates pre-coated with a 0.1 mg/ml solution of poly-L-lysine. They were maintained in DMEM:F12 (1:1) plus 10% horse serum and 250 μg/ml Geneticin (Life Technologies, Inc.) at 33°C. To induce differentiation in cultures at about 80% confluence, culture medium was replaced with serum-free DMEM:F12 (3:1) containing 1% bovine serum albumin (Boehringer-Mannheim), 1 μg/ml transferrin, 5 μg/ml insulin, 6.3 ng/ml progesterone, and 16.1 μg/ml putrescine, and the temperature was raised to 39°C. Additional experimental supplements (described in the text) were introduced to the medium 2 days after differentiation was initiated and then included in subsequent feedings. In most experiments, cultures were examined 9 days after switching to differentiating conditions, that is 7 days after exposure to experimental conditions. Cultures were examined using a Zeiss Axiovert 100 inverted microscope. Digital images were captured using Northern Eclipse software (Empix Imaging, Inc.).

Western Blots

RN46A cultures were rinsed in phosphate buffered saline, pH 7.4 (PBS), solubilized in 1% sodium dodecyl sulfate, and insoluble material was removed by centrifugation. Protein (20 μg/lane) was electrophoresed through a 12% acrylamide gel, then blotted to Immobilon P membrane (Millipore Corp.) in a buffer containing 40 μM AMPSO and 20% methanol using a semi-dry blotting apparatus. The blots were blocked in a solution of 1% nonfat milk powder and 0.05% Tween 20, then exposed to anti-GAP-43 monoclonal antibody 9-1E12 (Schreyer and Skene, 1991) ascites fluid diluted 1:20,000 in blocking solution (30 min). The filters were washed in PBS, exposed to biotinylated-anti-mouse IgG diluted 1:5,000, then to avidin-peroxidase diluted 1:5,000 (30 min each), followed by PBS washes. Peroxidase activity was revealed by reacting in 0.05% H_2O_2 and 0.025% diaminobenzidine (10 min). Blots were scanned and digital images were created using Adobe Photoshop software.

Immunocytochemistry

Cultures grown in 96 well plates were rinsed with Hank's buffered salt solution (HBSS) then fixed in 100% methanol at −20°C (30 min), rinsed with PBS, and blocked with PBS containing 2% horse serum and 0.5% bovine serum albumin (1 hr) at 37°C. Antibody 9-1E12 was applied at a dilution of 1:5,000 in blocking solution (45 min), then the cultures were washed with PBS 3× 5 min. Antibody binding was developed using biotin-conjugated anti-mouse IgG (1:500, 30 min) and avidin-peroxidase (1:500, 30 min) with appropriate washes. Culture plates were reacted in PBS containing 0.05% H_2O_2 and 0.025% diaminobenzidine (10 min) to localize peroxidase activity, and examined using a Zeiss Axiovert 100 inverted microscope. Digital images were captured using Northern Eclipse software.

DNA Analysis

DNA content of RN46A cultures was measured by the method of Rago et al. (1990). Cultures grown in 96 well plates were rinsed with HBSS, frozen at −70°C, then thawed. Lysis was completed by incubating with deionized water, then refreezing at −70°C. An equal volume of buffer (10 mM Tris pH 7.5, 2 M NaCl, 1 mM EDTA) containing 20 μg/ml Hoechst 33258 fluorescent dye was added and fluorescence was measured on a Labsystems Fluoroscan II plate reader (excitation 355 nm, emission 460 nm). Each assay was performed in sextuplicate. A standard curve was constructed using salmon sperm DNA dissolved in water and processed in parallel with cultured cells.

DNA labeling was also used to carry out direct cell counts. Cultures were fixed in methanol, then treated with 0.1 μg/ml Hoechst 33258 in PBS and examined using a Zeiss Axiovert 100 inverted microscope equipped with fluorescence optics. Digital images were captured using Northern Eclipse software. The number of fluorescent RN46A cell nuclei was counted in randomly chosen fields. For each experimental condition, 17 fields were counted.

cAMP Analysis

cAMP was assayed using a radio-binding assay system (Amersham, TRK 432) according to the manufacturer's instructions. Cultures grown in 24 well-plates (Falcon) were rinsed with PBS and extracted twice with methanol (5 min each). Extracts were combined, evaporated to dryness, dissolved in binding buffer, combined with cAMP binding protein and 3H-cAMP, then incubated at 4°C (2 hr). Free nucleotide was removed by charcoal absorption and the radioactive labeling of the protein complex was measured using a Beckman LS 6500 liquid scintillation counter. cAMP was calculated in comparison to a standard curve. Each assay was performed in triplicate.

Cell-ELISA Quantification of GAP-43

The GAP-43 protein content of cultures in 96 well plates was measured using cell-ELISA (Schreyer et al., 1997). Cultures were rinsed with HBSS and fixed in 100% methanol at −20°C

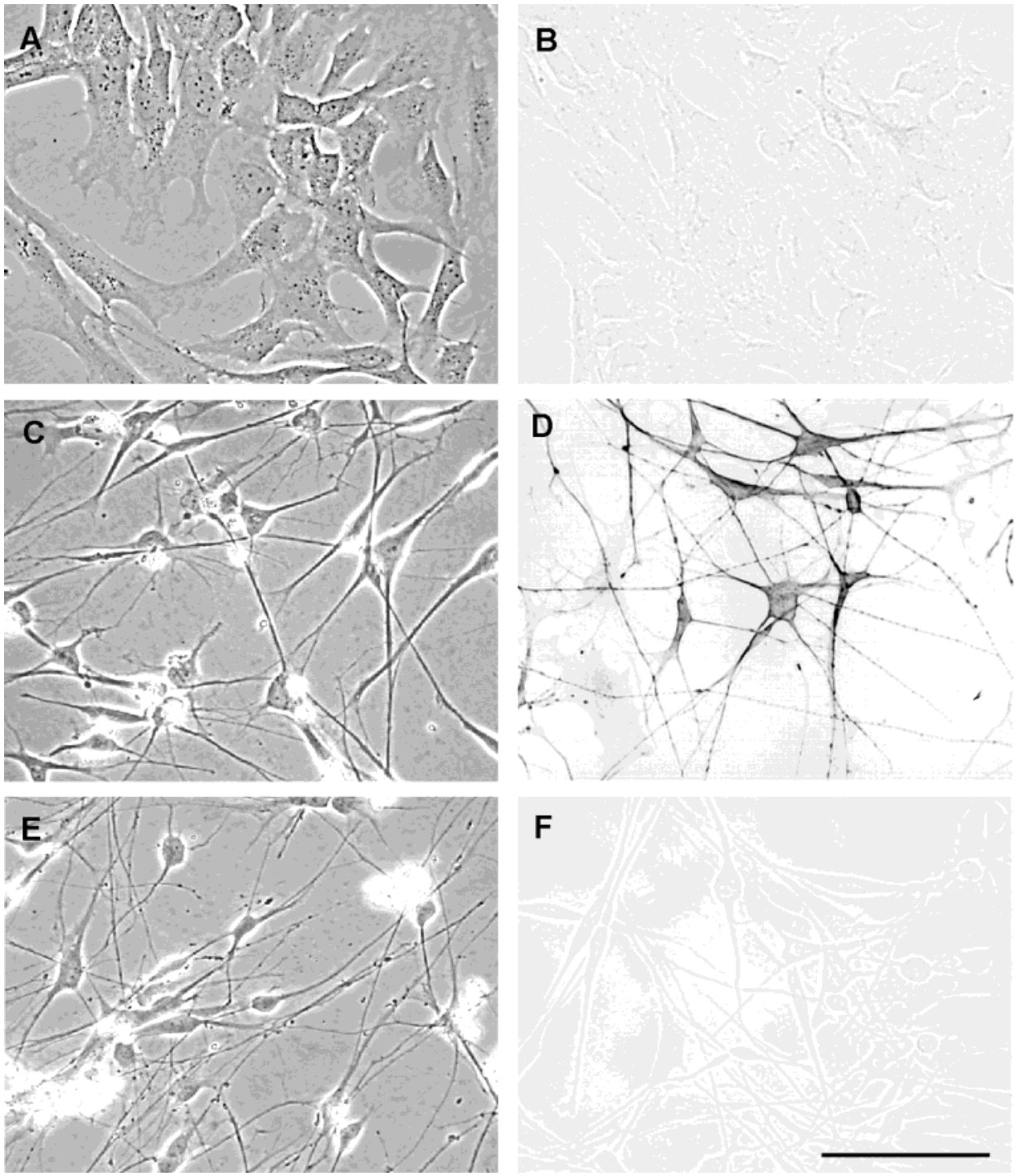

Fig. 1. (**A**) Phase contrast image of undifferentiated RN46A cells. (**B**) GAP-43 ICC of undifferentiated RN46A cells. (**C**) Phase contrast image of RN46A cells 9 days after differentiation. (**D**) GAP-43 ICC of RN46A cells 9 days after differentiation. (**E**) Phase contrast image of RN46A cells 9 days after differentiation and 7 days after exposure to 100 μM dBcAMP. (**F**) GAP-43 ICC of RN46A cells 9 days after differentiation and 7 days after exposure to 100 μM dBcAMP. Scale bar = 25 μm.

(30 min). Cultures were dried under vacuum, then treated with blocking solution consisting of PBS containing 10% nonfat dry milk powder and 0.5% Tween 20 at 37°C (1 hr). Blocking solution was replaced with blocking solution containing monoclonal antibody 9-1E12 against GAP-43 (Schreyer and Skene, 1991), ascites fluid diluted 1:5,000 at room temperature (45 min). Wells were washed 3× 5 min with PBS then incubated with horseradish peroxidase conjugated anti-mouse IgG

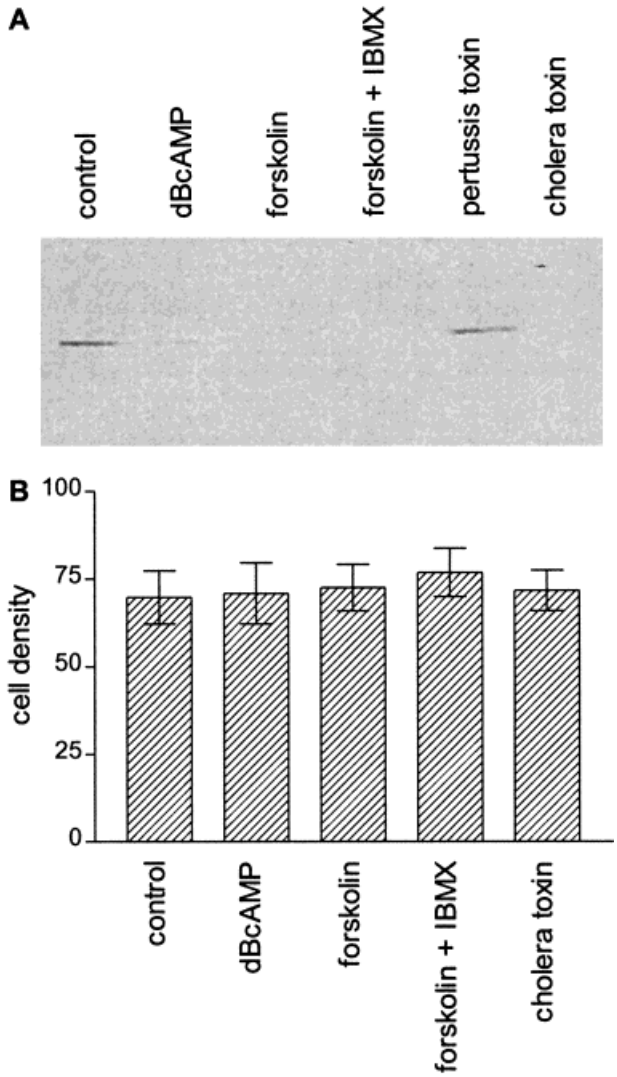

Fig. 2. (**A**) GAP-43 Western blot of differentiated RN46A cultures exposed for 7 days to 100 μM dBcAMP, 1 μM forskolin, 1 μM forskolin plus 50 μM IBMX, or 1 ng/ml cholera toxin. (**B**) Cell density counts of differentiated RN46A cultures exposed for 7 days to 100 μM dBcAMP, 1 μM forskolin, 1 μM forskolin plus 50 μM IBMX, or 1 ng/ml cholera toxin. Cell density did not vary significantly (ANOVA). Each bar represents the mean of 17 determinations made in one experiment. Error bars indicate SD. Results are representative of two independent experiments.

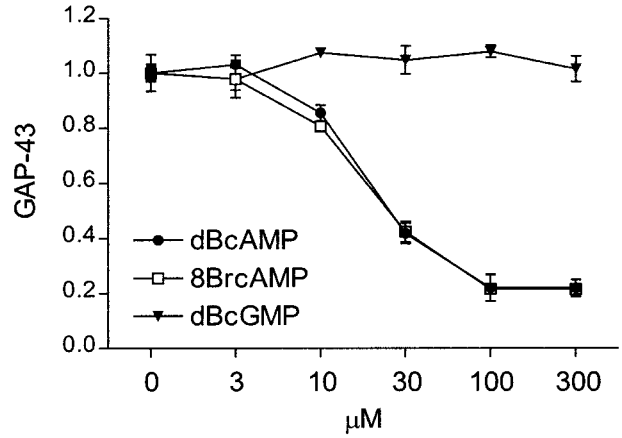

Fig. 3. Cell-ELISA measurement of GAP-43 (normalized to total DNA) in differentiated RN46A cultures exposed for 7 days to 0–300 μM dBcAMP, 8BrcAMP or dBcGMP. The decrease in GAP-43 with dBcAMP and 8BrcAMP was significant ($P < 0.001$; ANOVA followed by Tukey's comparison with control) at concentrations of 10 μM or greater. Each point represents the mean of 6 determinations made in 1 experiment. Error bars indicate SD. Results are representative of 7 independent experiments.

diluted 1:500 in blocking solution at room temperature (30 min). The wells were washed 3× 5 min with PBS and peroxidase activity was revealed by reacting with H_2O_2 and *o*-phenylene diamine dihydrochloride, supplied as a tablet set and used according to the manufacturer's instructions. The reaction was stopped by adding 3 M HCl and the absorbance in each well was read at 490 nm using a Spectromax 340 microplate reader. Each assay was carried out in sextuplicate. A standard curve was prepared on each plate in duplicate by applying partially purified GAP-43 (0–1.1 ng/well) in water to uncoated wells the night before the assay was performed, then processing those wells in parallel with the wells containing cultured cells.

Neurite Growth Potential Assay

RN46A cells were passaged to secondary culture 7 days after exposure to experimental conditions. Cultures were rinsed with HBSS and resuspended by lightly triturating approximately twelve times with a 200 μl micropipette tip. Cells were reseeded at approximately 25-fold lower density onto 35 mm poly-L-lysine-coated tissue culture plates. Cells were allowed to settle and extend processes for 8 hr, then fixed by adding glutaraldehyde directly to the culture medium to a concentration of 0.5% (30 min). Plates were rinsed with PBS then stained with 0.25% Coomassie blue in 45% methanol and 10% acetic acid (30 min) and rinsed with water. Cultures were examined using a Zeiss Axiovert 100 inverted microscope and neurite length was determined using a Northern Eclipse image analysis system according to the method of Jap Tjoen San et al. (1991). Images of the first 100 isolated cells encountered along a defined track were acquired and stored for analysis. Images were processed by 1) applying a threshold function to delineate cell bodies and processes, 2) 'erasing' cell bodies and debris, 3) applying a skeletonization function to convert neurites into one pixel diameter, and 4) summing remaining pixels to estimate total neurite length.

RESULTS

Appearance of Cultures

RN46A cells grown at 33°C displayed a flat, polygonal morphology and divided rapidly (Fig. 1A). When culture conditions were changed to 39°C and serum free medium, the cells ceased dividing and began to extend slender, sparsely branched neurites (Fig. 1C). Immunocytochemistry (ICC) indicated that undifferentiated RN46A

cells contained no detectable GAP-43 protein (Fig. 1B), but that after 9 days in differentiating conditions, GAP-43 expression was robust (Fig. 1D).

In subsequent experiments, we began exposure to experimental agents 2 days after switching cultures to differentiating conditions. This strategy was designed to test our ability to manipulate GAP-43 expression after differentiation to the neuronal phenotype had occurred. Differentiated RN46A cells were exposed to the membrane permeable cAMP analog 100 μM dibutyryl cyclic AMP (dBcAMP) for 7 days. RN46A cells continued to display a differentiated morphology and slender neurites were still prominent (Fig. 1E). GAP-43 ICC, however, indicated that the presence of this protein was dramatically reduced (Fig. 1F).

To confirm that antibody 9-1E12 was recognizing authentic GAP-43 in our cultures, we carried out Western blot analysis of proteins extracted from RN46A cells that had been differentiated, then grown in experimental conditions for 7 days. The antibody labeled a single band previously characterized as GAP-43 (Schreyer and Skene, 1991). The intensity of this band was decreased in cultures treated with 100 μM dBcAMP, and also with adenyl cyclase activator 1 μM forskolin, with 1 μM forskolin plus phosphodiesterase inhibitor 50 μM isobutylmethylxanthene (IBMX), or with 1 ng/ml cholera toxin, that activates adenyl cyclase indirectly by ADP-ribosylating the adenyl cyclase stimulating G-protein G_s (Fig. 2A). Pertussis toxin, another ADP ribosyl transferase that inactivates other G-proteins, including G_i, had no effect.

To confirm that loss of GAP-43 seen in ICC and Western blot experiments was not due to RN46A cell loss, we counted Hoechst-labeled cell nuclei in cultures of the same age. Treatment of cultures with 100 μM dBcAMP, 1 μM forskolin, 1 μM forskolin plus 50 μM IBMX, or 1 ng/ml cholera toxin caused no significant change in RN46A cell number (Fig. 2B).

Quantification of Effect of cAMP Analogs on GAP-43

We used cell-ELISA to measure GAP-43 protein 7 days after exposure to dBcAMP or 8-bromo cyclic AMP, another membrane permeable analog of cAMP.

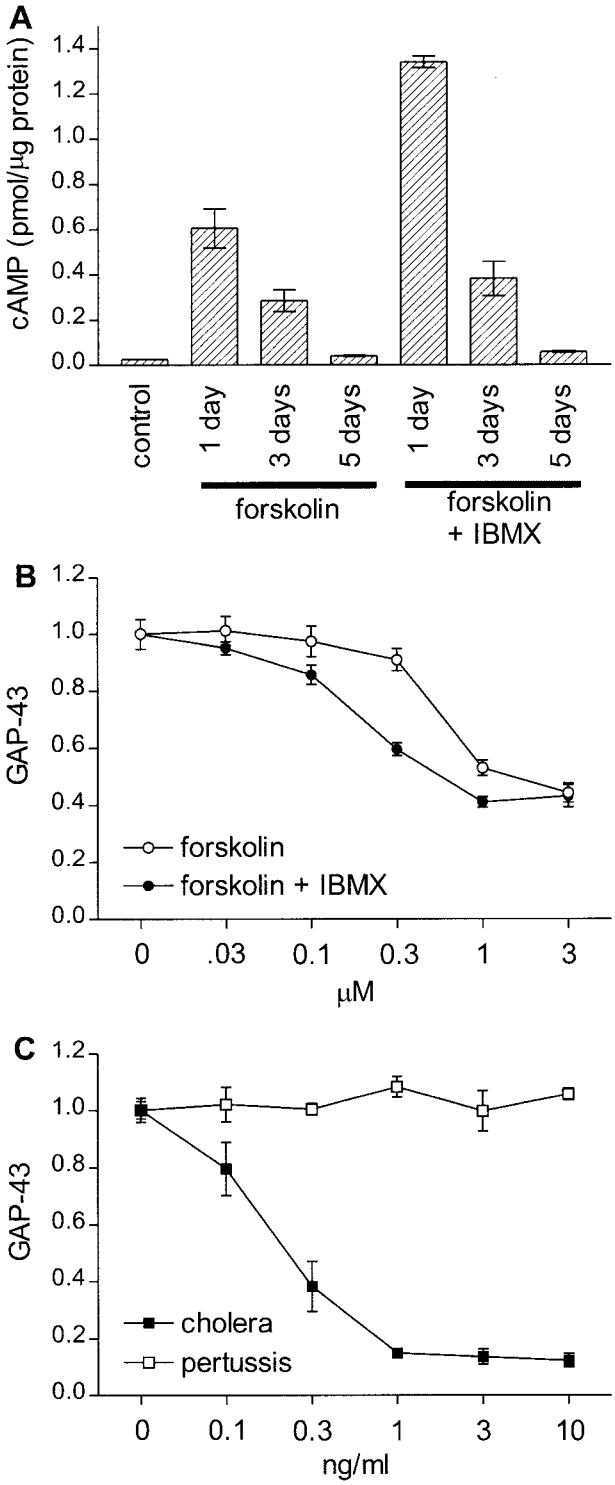

Fig. 4. (**A**) Radio binding assay for cAMP (normalized to total protein) in differentiated RN46A cultures exposed for 1, 3, or 5 days to 1μM forskolin, 1μM forskolin plus 50 μM IBMX, or 1 ng/ml cholera toxin. Significant ($P < 0.001$; ANOVA followed by Tukey's comparison with control) increases were seen at 1 and 3 days exposure to all experimental conditions. Each bar represents the mean of 3 determinations made in 1 experiment. Error bars indicate SD. Results are representative of 5 independent experiments. (**B**) Cell-ELISA measurement of GAP-43 (normalized to DNA) in differentiated RN46A cultures exposed for 7 days to 0–10 μM forskolin, with or without 50 μM IBMX. The decrease in GAP-43 was significant ($P < 0.001$; ANOVA followed by Tukey's comparison with control) at forskolin concentrations of 0.3 μM or greater, and at forskolin concentrations of 0.1 or greater when IBMX was present. Each point represents the mean of 6 determinations made in 1 experiment. Error bars indicate standard deviation. Results are representative of 3 independent experiments. (**C**) Cell-ELISA measurement of GAP-43 (normalized to DNA) in differentiated RN46A cultures exposed for 7 days to 0–10 ng/ml cholera toxin or 0–10 ng/ml pertussis toxin. The decrease in GAP-43 was significant ($P < 0.001$; ANOVA followed by Tukey's comparison with control) at cholera toxin concentrations of 0.1 ng/ml or greater. Each point represents the mean of 6 determinations made in one experiment. Error bars indicate SD. Results are representative of 6 independent experiments.

With both reagents, GAP-43 content of RN46A cells, normalized to DNA content, was decreased in a dose dependent manner (Fig. 3). DNA content did not vary significantly (not separately shown). With both analogs, GAP-43 was decreased by 80% at 100 μM. Dibutyryl cyclic GMP did not alter GAP-43 protein levels at any concentration tested.

Endogenous cAMP Decreases GAP-43 Protein Expression

To determine the effects of physiological attainable levels of cAMP, differentiated RN46A cultures were chronically exposed to forskolin, with or without IBMX, and to cholera toxin. A cAMP radio binding assay was used to show that forskolin caused a rapid, large increase in cAMP in differentiated RN46A cells (Fig. 4A). Although this effect declined with continuing exposure, significant elevation of cAMP persisted for at least 3 days. The effect of forskolin was potentiated by co-administration of IBMX. Similarly, cholera toxin caused a rapid increase in cAMP, decrementing with continued exposure (not shown).

Forskolin at 1 μM caused a decrease in the GAP-43 content of RN46A cultures by 50% and this effect was augmented to a 60% decrease in the presence of 50 μM IBMX (Fig. 4B), with no loss of DNA content (not separately shown). GAP-43 protein content of RN46A cultures was reduced by 80% after chronic exposure to 1 ng/ml cholera toxin (Fig. 4C). DNA content was not affected (not separately shown). Pertussis toxin up to 100 ng/ml had no effect on GAP-43 in RN46A cultures (Fig. 4C).

To confirm that cholera toxin repression of GAP-43 was mediated by adenyl cyclase, cultures were chronically exposed to 1 ng/ml cholera toxin in the presence of the adenyl cyclase inhibitor SQ22,536 (RBI). This inhibitor blocked the ability of cholera toxin to repress GAP-43 in a dose dependent manner, but had no effect on GAP-43 when used alone (Fig. 5A). We also examined the effects of a competitive inhibitor of protein kinase A, dideoxyadenosine (DDA). Cultures chronically exposed to 10 μM DDA showed an increase in GAP-43 protein of approximately 50%. DDA could also partially reverse the repressive effects of cholera toxin (Fig. 5B). DDA applied at 100 μM or above significantly reduced neuronal survival (not shown).

We also used cell-ELISA to determine if potential target derived growth factors could act similarly to cAMP and down-regulate GAP-43 as part of a maturation influence. We found that the neurotrophins NGF (Cedar Lane Laboratories), BDNF (Alomone Labs), NT-3 (Alomone Labs) and NT-4 (Alomone Labs) had no effect on GAP-43 content of differentiated RN46A cultures at doses up to 100 ng/ml (not shown). Similarly, the cytokines CNTF (Alomone Labs), LIF, and GDNF and the growth factor IGF1 had no effect at concentrations up to 100 ng/ml (not shown).

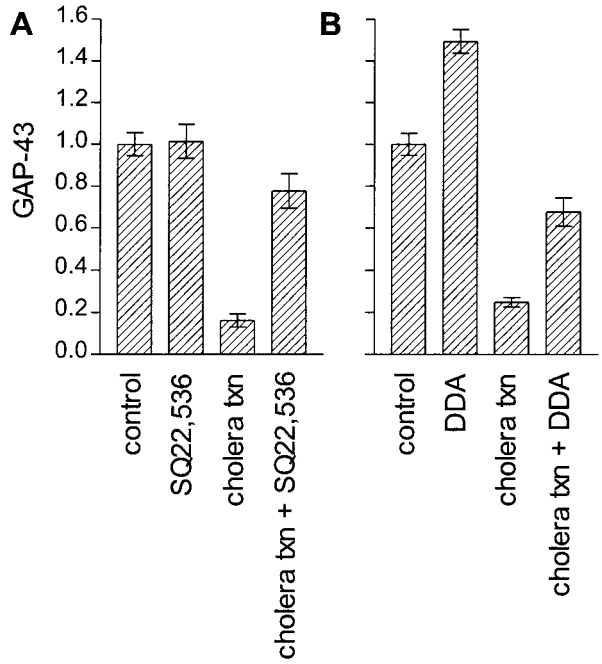

Fig. 5. (**A**) Cell-ELISA measurement of GAP-43 (normalized to total DNA) in differentiated RN46A cultures exposed for 7 days to 1 ng/ml cholera toxin and 100 μM SQ22,536, alone or in combination. SQ22,536 alone had no significant effect on GAP-43, but significantly ($P < 0.001$; ANOVA followed by Tukey's comparison with cholera toxin alone) reversed the repressive effect of cholera toxin. Each bar represents the mean of 6 determinations made in 1 experiment. Error bars indicate SD. Results are representative of 4 independent experiments. (**B**) Cell-ELISA measurement of GAP-43 (normalized to total DNA) in differentiated RN46A cultures exposed for 7 days to 1 ng/ml cholera toxin and 10 μM DDA, alone or in combination. DDA alone caused a significant ($P < 0.001$; ANOVA followed by Tukey's comparison with control) increase in GAP-43, and significantly ($P < 0.001$; ANOVA followed by Tukey's comparison with cholera toxin alone) reversed the repressive effect of cholera toxin. Each bar represents the mean of 6 determinations made in 1 experiment. Error bars indicate SD. Results are representative of 3 independent experiments.

cAMP Enhances Neurite Extension

Neurite outgrowth seemed to be enhanced in standard density RN46A cultures during the first 12 hr after they were exposed to dBcAMP, forskolin, or cholera toxin (not shown). Seven days after treatment, however, all control and experimental cultures of differentiated RN46A cells displayed extensive neurite outgrowth (Fig. 1C,E), and neuritic arbors could not be identified individually and quantified morphometrically under these conditions.

To examine neurite growth potential at the 7-day time point, rather than net neurite outgrowth at the end of 7 days, RN46A cells were resuspended by gentle trituration and plated into secondary culture at lower density. This procedure resulted in loss of pre-existing neurites (Fig. 6A) and the cells attached to the substratum within 30 min. The extent of neurite growth could then be investigated in these secondary cultures during an 8-hr test

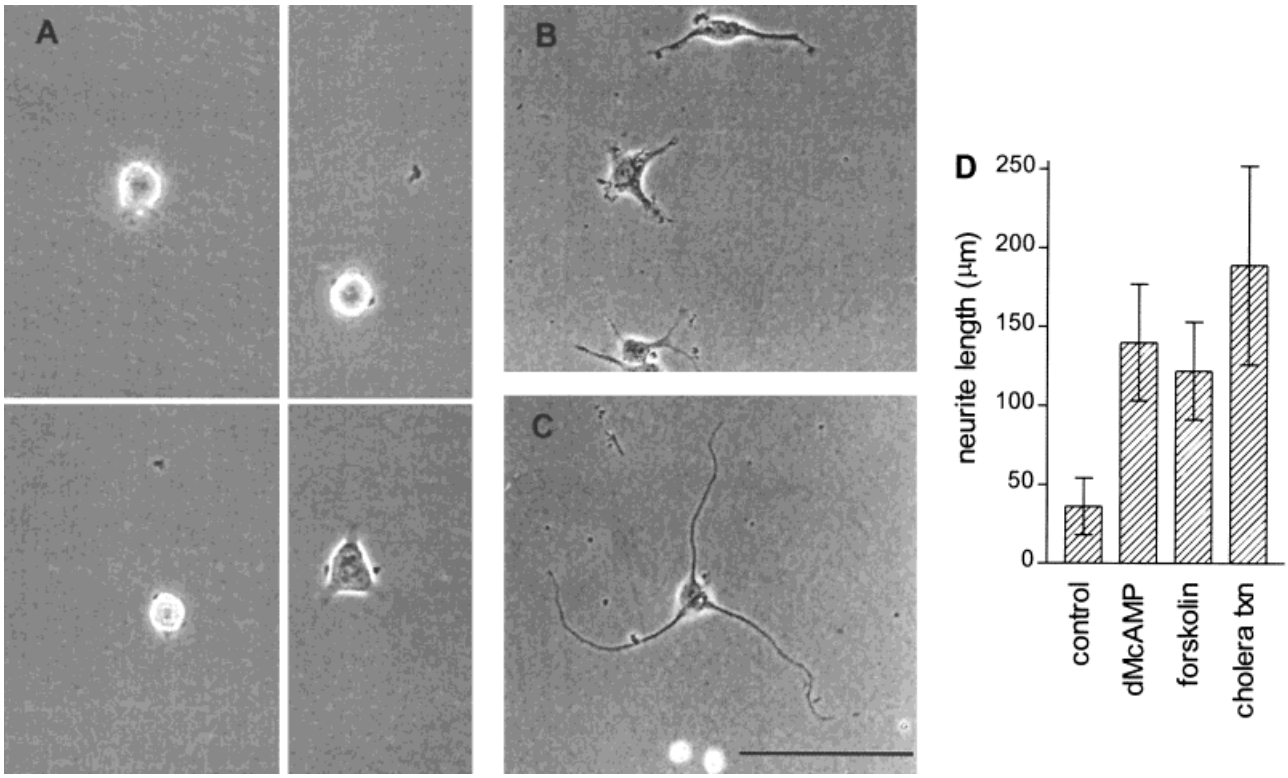

Fig. 6. (**A**) Phase contrast image of RN46A cells acutely passaged into secondary culture 9 days after differentiation. (**B**) Phase contrast image of RN46A cells 8 hr after passaging into secondary culture with exposure to control conditions at all times. (**C**) Phase contrast image of RN46A cell 8 hr after passaging into secondary culture containing 1 ng/ml cholera toxin, after 7 days previous exposure to 1 ng/ml cholera toxin. Bar represents 25 μm. (**D**) Measurement of mean neurite length per neuron in RN46A cultures 8 hr after passaging into secondary culture, with 7 days previous exposure, and continuing exposure, to control conditions, 100 μM dBcAMP, 1 μM forskolin, or 1 ng/ml cholera toxin. All experimental treatments caused a significant ($P < 0.001$; ANOVA followed by Tukey's comparison with control) increase in neurite length. Each bar represents the mean of 121 determinations made in 1 experiment. Error bars indicate SD. Results are representative of 4 independent experiments.

period. RN46A cells receiving pre-exposure and test exposure to 1 ng/ml cholera toxin displayed neurites that were, on average, at least 3 times as long after 8 hr as those seen in control cultures (Fig. 6B,C). Similar results were obtained in cultures that were exposed to 100 μM dB-cAMP or 10 μM forskolin before and after passaging into the secondary test culture (Fig. 6D).

Under all conditions of normal or stimulated growth during the initial 7-day culture period and during the subsequent 8 hr secondary culture, neurites were slender, and the great majority (>85%) displayed either 0, 1, or 2 branch points per neurite.

Effect of cAMP on Neurite Growth Is Rapid and Reversible

To determine whether the effect of cAMP signaling on neurite extension was rapid, RN46A cells grown for 7 days with or without 1 ng/ml cholera toxin were passaged to low density into medium with or without 1 ng/ml cholera toxin. New neurite outgrowth from RN46A cells previously exposed to control conditions was enhanced if these cells were switched to medium containing cholera toxin for eight hours in secondary culture. Conversely, cells chronically exposed to cholera toxin extended significantly longer new neurites in secondary culture only if they remained in cholera toxin medium, and not if they were switched into control medium (Fig. 7). Thus, neurite extension is potentiated by cholera toxin in secondary culture independently of the prior history of cholera toxin exposure and suppression of GAP-43 protein expression.

We then examined whether cholera toxin stimulation of neurite growth requires new transcription. Exposure of cultures to the transcription inhibitor 80 μM DRB reduced incorporation of radiolabeled uridine into RNA by >95% in differentiated RN46A cultures (Fig. 8A). Seven day cultures were then replated and exposed to 1 ng/ml cholera toxin with or without 80 μM DRG, during an 8 hr neurite growth test period. Neither control neurite outgrowth nor stimulation of neurite outgrowth by cholera toxin was affected by inclusion of 80 μM DRB (Fig. 8B), suggesting that enhancement of neurite outgrowth during the test period was not dependent on rapid synthesis of new GAP-43, or any other protein.

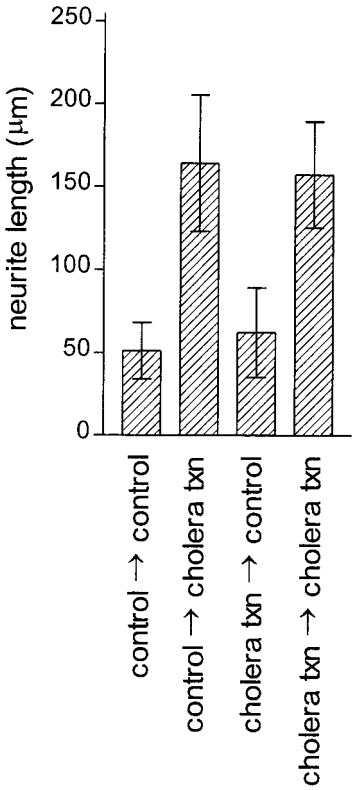

Fig. 7. Measurement of mean neurite length per neuron in RN46A cultures maintained for 7 days either with or without 1 ng/ml cholera toxin, then passaged into secondary culture for 8 hr either with or without 1 ng/ml cholera toxin. There was a significant ($P < 0.001$; ANOVA followed by Tukey's comparison with control) increase in neurite length in secondary cultures exposed to cholera toxin, whether or not they had 7 days previous exposure to cholera toxin. Previous exposure to cholera toxin had no significant effect on cultures grown in control conditions in secondary culture. Each bar represents the mean of 120 determinations made in 1 experiment. Error bars indicate SD. Results are representative of 4 independent experiments.

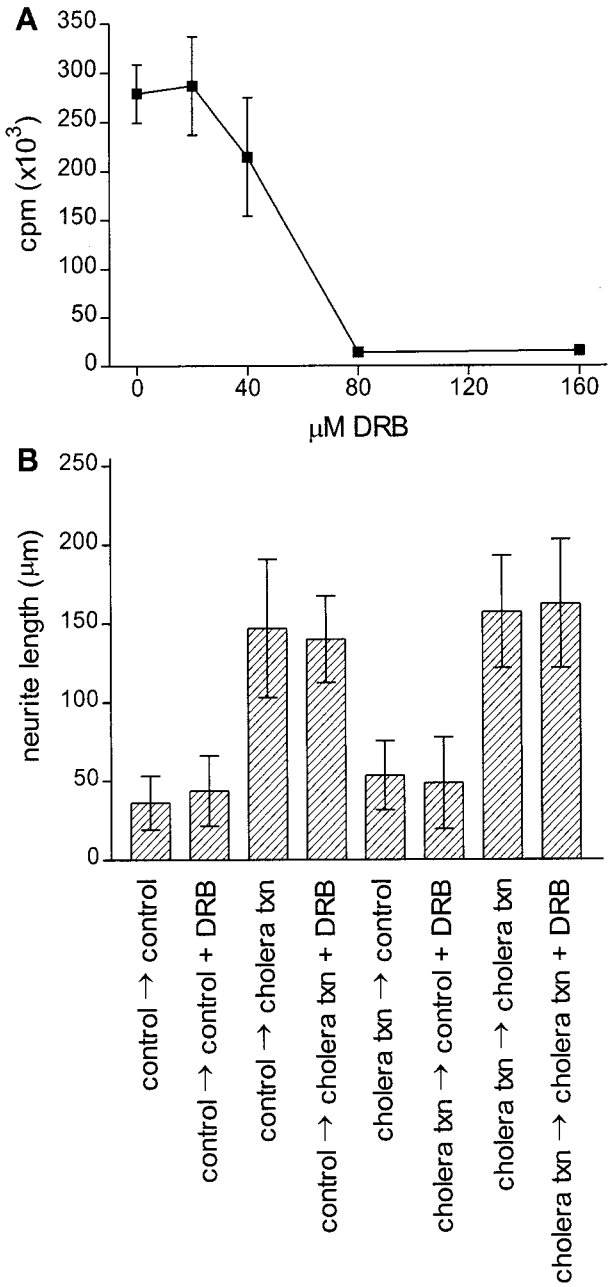

Fig. 8. (**A**) Incorporation of tritiated uridine into RNA in differentiated RN46A cultures exposed for 2 hr to 0–160 μM DRB. Uridine incorporation was decreased to less than 5% of control at DRB concentrations of 80 μM or greater. (**B**) Measurement of mean neurite length per neuron in RN46A cultures maintained for 7 days in control conditions or 1 ng/ml cholera toxin, then passaged into secondary culture for 8 hr with or without 1 ng/ml cholera toxin and with or without 80 μM DRB. DRB had no effect on neurite length in control cultures, and could not block the significant ($P < 0.001$; ANOVA followed by Tukey's comparison with control) stimulation of neurite growth by cholera toxin. This was true whether the cultures received 7 day pre-exposure to control conditions (high GAP-43 pre-expression, see Fig. 4C) or to 1 ng/ml cholera toxin (low GAP-43 pre-expression, see Fig. 4C). Each bar represents the mean of 100 determinations made in 1 experiment. Error bars indicate SD. Results are representative of 3 independent experiments.

DISCUSSION

GAP-43 protein in RN46A cells could not be detected while the cells were undifferentiated and undergoing proliferation. After they were switched to serum-free differentiating culture conditions, they ceased dividing, extended neurites, and expressed high levels of GAP-43. Thus, process outgrowth and high GAP-43 expression seem to be intrinsic features of the neuronal phenotype of RN46A cells.

cAMP and Neuronal Maturation

Our finding that activation of cAMP signaling causes a down-regulation of GAP-43 expression in differentiated RN46A cells is consistent with previous observations that cAMP down-regulates GAP-43 in DRG neurons (Schreyer et al., 1997; Andersen et al., 2000) and Schwann cells (Scherer et al., 1994). Thus, this GAP-43 repressive pathway seems to be shared by several nervous system cell types.

Raphe neurons developing in vivo seem to undergo a decrease in GAP-43 expression after they establish contact with their targets (Dani et al., 1991). Substantial GAP-43 expression in Raphe neurons, however, persists into adulthood (Bendotti et al., 1991). Our results using ICC and Western blotting suggest a near total loss of GAP-43 immunoreactivity in RN46A cells after activation of cAMP signaling, but our more quantitative cell-ELISA technique indicates that GAP-43 protein in these cells drops to approximately 20% of control. Thus, for both putative target-derived repression in vivo and cAMP-induced repression in vitro, GAP-43 expression is apparently not eliminated entirely.

The cAMP signaling pathway seems to be involved in establishing several prominent features of neuronal maturation. RN46A cells increase their synthesis of neurofilament proteins upon activation of cAMP signaling (White et al., 1997). Moreover, both ACTH, acting through adenyl cyclase, (Eaton and Whittemore, 1995) and BDNF (Eaton et al., 1995) interact with depolarization to increase expression of the serotonergic properties of RN46A cells. These effects are also seen in primary cultures of raphe neurons (Azmitia et al., 1990; Eaton and Whittemore, 1995; Suiciak et al., 1994, 1998). Here we show that activation of adenyl cyclase also causes down-regulation of GAP-43, opposite to its effect on expression of neurofilament proteins and serotonergic markers, and consistent with the concept of a global switch to a more mature phenotype.

A target-derived regulator of GAP-43 expression in Raphe neurons has not been identified. The observation that cholera toxin down-regulates GAP-43, however, indicates that the heterotrimeric G-protein Gs is present, and invites the inference that receptors linked to Gs may be present and may serve to transduce an extracellular GAP-43-repressing signal. Although neurotrophins have previously been characterized as retrograde regulators of neuronal gene expression (Fitzgerald et al., 1985; Verge et al., 1990, 1995, 1996), we were unable to find an effect on GAP-43 expression after exposure to several neurotrophins, including BDNF. It should be noted that we did not combine exposure to neurotrophic factors with experimental depolarization, and depolarization may be a necessary adjunct for the full effect of neurotrophins in other systems (Cohen-Corey et al., 1991; Diamond et al., 1992; Eaton et al., 1995).

cAMP and Neurite Outgrowth

In that the acquisition of a mature phenotype in neurons corresponds with cessation of long axon growth, we were surprised to observe that cAMP caused an increase in neurite outgrowth, even while it repressed expression of GAP-43. Neurite outgrowth was stimulated by cAMP signaling within 12 hr in newly differentiated RN46A cell cultures. With continuing exposure, neurite growth in standard density control and stimulated cultures became so extensive as to preclude recognition of a difference. If cultures were resuspended after 7 days and replated at low density, however, new neurite outgrowth was again stimulated upon activation of cAMP.

The effect of cAMP on neurite outgrowth seemed to be rapidly reversible. When long-term control or cholera toxin-treated cultures were shorn of neurites and replated, new neurite outgrowth was rapidly potentiated by cholera toxin, even in cells never previously exposed to cholera toxin. This effect was also seen in the presence of a transcription inhibitor, suggesting that the effect of cholera toxin on neurite outgrowth is not mediated by changes in gene expression. Rather, the motility apparatus within the growth cone is likely to be directly responsive to local cAMP signaling (Ellis et al., 1985; Lohof et al., 1992; Song et al., 1997).

Interestingly, enhanced growth of neurites in response to cAMP bears no relation to their existing GAP-43 content. Thus, RN46A cells depleted of GAP-43 by prolonged exposure to cholera toxin could still respond with enhanced neurite growth when exposure to cholera toxin was continued in secondary culture, even in the presence of transcription inhibitors. Thus, growth cone responsiveness to cAMP apparently does not require the signal transducing properties of GAP-43. Interestingly, specific enhancement of process outgrowth by the neural cell adhesion molecule seems to require the presence of GAP-43 (Meiri et al., 1998), indicating that GAP-43 does participate in transducing the signal from at least one positively acting extracellular growth regulator.

Summary

Although our studies do not identify target derived regulators of GAP-43 expression and axon growth, they identify a signaling pathway that could mediate the action of GAP-43 repressive influences originating extracellularly. Neither this signaling pathway nor any parallel cAMP signaling pathway, however, seems to repress growth. Indeed, cAMP acts to stimulate neurite outgrowth in a manner that is epigenetic and independent of altered synthesis or presence of GAP-43.

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